REPORT

SILRES® BS 1701: Toxicity to *Pseudokirchneriella subcapitata* in a 72-Hour Algal Growth Inhibition Test

Study Director: Dr. S. Höger

Test Facility: **Harlan Laboratories Ltd.**
Zelgliweg 1
4452 Itingen / Switzerland

Sponsor: **Wacker Chemie AG**
Johannes-Hess-Str. 24
84489 Burghausen / Germany

Study Identification: Harlan Laboratories Study **C17025**

Version: Final

Study Completion Date: 17-Aug-2010

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GLP CERTIFICATE

The Swiss GLP Monitoring Authorities

Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra

Swiss Confederation

Federal Department of Home Affairs DHA
Federal Office of Public Health FOPH

Federal Department of the Environment, Transport, Energy and Communications DETEC
Federal Office for the Environment FOEN

swissmedic
Swiss Agency for Therapeutic Products

Statement of GLP Compliance

According to Article 14 paragraph 3 Ordinance on Good Laboratory Practice [OGLP, SR 813.112.1]

The notification authority for chemicals confirms that the following test facility was inspected with respect to the compliance with the Swiss Ordinance on Good Laboratory Practice, adopted on 18th May 2005 [OGLP, SR 813.112.1]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted on 26th November 1997 by decision of the OECD Council [C(97)186/Final].

Unequivocal name and address of the test facility:	Areas of expertise according to article 3 paragraph 1 letter d OGLP:
Harlan Laboratories Ltd. Zelgliweg 1 4452 Itingen, Switzerland.	1./ Physical-chemical testing, 2./ Toxicity studies, 4./ Environmental toxicity studies on aquatic and terrestrial organisms, 5./ Studies on behaviour in water, soil and air; bioaccumulation, 6./ Residue studies, 7./ Studies on effects on mesocosms and natural ecosystems, 8./ Analytical and clinical chemistry testing, 9./ Other studies (safety pharmacology and animal metabolism).

Inspection authority: Federal Office for the Environment (FOEN) / Federal Office of Public Health (FOPH) / Swiss Agency for Therapeutic Products (Swissmedic)

Date of inspection: 05th to 09th and 26th to 30th November 2007

Date of decision: 30th April 2008

Based on the above mentioned decision it can be confirmed that the above mentioned test facility is able to conduct studies according to the aforementioned areas of expertise in compliance with the principles of GLP. The above mentioned test facility is listed in the register and GLP list according to the Article 14 OGLP and is inspected on a regular basis according to Article 6 paragraph 2 OGLP.

Swiss Federal Office of Public Health
Consumer protection directorate
Notification authority for chemicals
CH-3003 Bern

Dag Kappes

FEDERAL OFFICE OF PUBLIC HEALTH

Bern, 12th November 2008, The Head, Dr. Dag Kappes.

The notification authority for chemicals is the coordination and decision authority for the good laboratory practice (GLP) for the FOEN, the FOPH and Swissmedic.

Swiss Federal Office of Public Health, Consumer protection directorate, Notification authority for chemicals, CH-3003 Bern.

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GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE

Harlan Laboratories Study:	C17025
Test Item:	SILRES® BS 1701
Study Director:	Dr. S. Höger
Study Title:	SILRES® BS 1701: Toxicity to *Pseudokirchneriella subcapitata* in a 72-hour algal growth inhibition test

This study has been performed in compliance with the Swiss Ordinance relating to Good Laboratory Practice, adopted May 18th, 2005 [SR 813.112.1]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26th, 1997 by decision of the OECD Council [C(97) 186/Final].

These principles are compatible with Good Laboratory Practice regulations specified by regulatory authorities throughout the European Community, the United States (EPA and FDA), and Japan (MHLW, MAFF and METI).

Exclusions:

- Pre-experiments as mentioned in the report.

There were no circumstances that may have affected the quality or integrity of the data.

Study Director: Dr. S. Höger

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Date: AUGUST 17, 2010

QUALITY ASSURANCE STATEMENT

Harlan Laboratories Ltd., Zelgliweg 1, 4452 Itingen / Switzerland

Harlan Laboratories Study:	C17025
Test Item:	SILRES® BS 1701
Study Director:	Dr. S. Höger
Study Title:	SILRES® BS 1701: Toxicity to *Pseudokirchneriella subcapitata* in a 72-hour algal growth inhibition test

The general facilities and activities are inspected at least once a year and the results are reported to the responsible person and the management.

Study procedures were periodically inspected. The study plan and this report were audited by the Quality Assurance. The dates are given below.

Dates and Types of QA Inspections		Dates of Reports to the Study Director and to the Test Facility Management
03-Sep-2009	Study Plan	03-Sep-2009
12-Jan-2010	Process based (test system)	12-Jan-2010
12-Jan-2010	Process based (work up)	12-Jan-2010
30-Mar-2010	Appendix I – Analytical Investigations	01-Apr-2010
30-Mar-2010	Report	01-Apr-2010

This statement also confirms that this final report reflects the raw data.

Quality Assurance: Ms. D. Leiminer

D. Leiminer
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Date: August 16, 2010

SIGNATURE OF ADDITIONAL SCIENTIST

Analytical Chemistry: Dr. K. Stoob

Dr. Ch. Zepperitz

Date: August 17, 2010

PREFACE

General Information

Test Item:	SILRES® BS 1701
Study Title:	SILRES® BS 1701: Toxicity to *Pseudokirchneriella subcapitata* in a 72-hour algal growth inhibition test
Sponsor:	Wacker Chemie AG Johannes-Hess-Str. 24 84489 Burghausen / Germany
Study Monitor:	Dr. A. Bosch
Test Facility:	Harlan Laboratories Ltd. Zelgliweg 1 4452 Itingen / Switzerland
QA:	Harlan Laboratories Ltd. Quality Assurance GLP Zelgliweg 1 4452 Itingen / Switzerland

Responsibilities

Study Director:	Dr. S. Höger
Analytical Chemistry:	Dr. K. Stoob
Head of QA:	Mr. T. Fink

Schedule

Experimental Starting Date:	04-Dec-2009
Experimental Completion Date:	20-Jan-2010

Data Requirements / Test Guidelines

The study was performed in accordance with the procedures indicated by the following internationally accepted guidelines and recommendations:

- OECD Guidelines for the Testing of Chemicals, No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test, 2006.
- EU Commission Directive 92/69/EEC, C.3: Algal Inhibition Test, 1992.
- Commission Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), C.3: Algal Inhibition Test.

Summary of Study Plan Amendments

There were no amendments to the Study Plan.

Archiving

Harlan Laboratories Ltd. (4452 Itingen / Switzerland) will retain the study plan, raw data, a sample of the test item and the final report of the present study for at least ten years. No data will be discarded without the Sponsor's written consent.

DEFINITIONS

Biomass:	Measurement variable (fluorescence) as surrogate measure for algal biomass.
Growth:	The increase of biomass over the test period.
Growth Rate (Average Specific Growth Rate):	The logarithmic increase in biomass during the exposure period.
Yield:	The value of the measurement variable at the end of the exposure period minus the measurement variable's value at the start of the exposure period, calculated to express biomass increase during the test.
EL_x:	The calculated loading rate that results in an x% reduction of the respective growth parameter relative to the control (or solvent control, if applicable).
NOELR (No Observed Effect Loading Rate):	The highest loading rate at which no statistically significant inhibition of growth is determined relative to the control (or solvent control, if applicable).
LOELR (Lowest Observed Effect Loading Rate):	The lowest loading rate at which a statistically significant inhibition of growth is determined relative to the control (or solvent control, if applicable).
Treatment:	Comprises test item treatments, control (test medium only) and solvent control (if applicable).

1 SUMMARY

The influence of the test item SILRES® BS 1701 on the growth of the freshwater green algal species *Pseudokirchneriella subcapitata* was investigated in a 72-hour static test according to OECD Guideline 201 (2006), the EU Commission Directive 92/69/EEC, C.3 (1992) and the Commission Regulation (EC) No 440/2008, C.3.

A dispersion of the test item was prepared in the test water and stirred for 96 hours in order to dissolve a maximum amount of the test item in the dispersion. After the stirring period, the dispersion was filtered through a membrane filter and the undiluted filtrate (saturated solution) was used as highest test concentration and as stock solution for the preparation of the lower concentrated test media.

The following treatments were tested: Dilution 1:100, 1:32, 1:10, 1:3.2 and the undiluted filtrate with a loading rate of 100 mg/L in parallel to a control.

At the start of the test, the analytically determined concentrations of the test item in the test media (dilution 1:3.2 and the undiluted filtrate) were 280 and 1200 µg/L, respectively. At the end of the test, the measured concentrations were both lower than the biological limit of quantification of the test item (LOQ_{bio}=10.1 µg test item/L). However, as the algae were exposed to the test item and all degradation products (produced during stirring time and the exposure period) the biological results were based on the loading rate of 100 mg/L and the dilutions thereof.

The test item had a significant inhibitory effect on the growth of the algae (average growth rate and yield) after the test period of 72 hours at the loading rate of 100 mg/L (undiluted filtrate). Thus, this loading rate was determined to be the 72-hour LOELR.

The 72-hour NOELR was determined to be dilution 1:3.2 of the loading rate of 100 mg/L, since up to and including this mean measured test concentration, the growth rate and yield of the algae after 72 hours were not significantly lower than in the control.

The biological results were summarized as follows:

Parameter (0-72 h)	**Growth rate**	**Yield**
EL_{50}	>100 mg/L	
NOELR	dilution 1:3.2 (of filtrate 100mg/L)	
LOELR	100 mg/L (loading rate)	

As the water solubility was determined to be <0.25 mg/l and the formation of colloidal fractions in the undiluted filtrate can not be excluded, the highest loading rate tested (100 mg/L) could be above the water solubility of the test item. However, due to the characteristics of the test item, the applied test design could not be improved further.

2 PURPOSE

The purpose of this test was to determine the effects of the test item SILRES® BS 1701 on the growth of the freshwater green algal species *Pseudokirchneriella subcapitata*. Exponentially growing cultures of this algal species were exposed to the test item over a period of 72 hours and the inhibition of algal growth in relation to control cultures was assessed over several generations.

3 MATERIALS AND METHODS

3.1 Test Item

The test item and the following information were provided by the Sponsor:

Identity:	SILRES® BS 1701
Batch No.:	KH07241
Purity:	94.1% (GC; 11.08.2008)
Expiration Date:	10-Oct-2010
Aggregate State / Physical Form at Room Temperature:	Liquid
Color:	Colorless
Storage Conditions:	Protect against moisture. Keep container tightly closed and store in a cool, well ventilated place. (At Harlan Laboratories: In the closed container at room temperature at about 20 °C, away from direct sunlight.)

3.2 Analytical Standard

The test item was used as analytical standard.

3.3 Test System

The test organism used for the study was *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*), Strain No. 61.81 SAG, supplied by the Collection of Algal Cultures (SAG, Institute for Plant Physiology, University of Göttingen, 37073 Göttingen / Germany). The algae were cultivated at Harlan Laboratories under standardized conditions according to the test guidelines.

Nygaard et al. [1] recommended describing the taxa within the Genus *Raphidocelis* HINDAK as:

Raphidocelis subcapitata (KORSIKOV) nov. comb.
Basionym: *Ankistrodesmus subcapitatus* KORSIKOV
Syn.: *Kirchneriella subcapitata* KORSIKOV
Syn.: *Selenastrum capricornutum* PRINTZ
Syn.: NIVA-CHL 1

An inoculum culture was set up four days before the start of the exposure. The algae were cultivated under the test conditions. The inoculum culture was diluted threefold one day before the start of the test to ensure that the algae were in the exponential growth phase when used to inoculate the test solutions.

For evaluation of the algal quality and experimental conditions, potassium dichromate is tested as a positive control twice a year to demonstrate satisfactory test conditions. The result of the latest positive control test performed in September 2009 showed that the sensitivity of the test system was within the internal historical range (72-hour EC_{50} for the growth rate: 0.75 mg/L (study C63678), range of the 72-hour EC_{50} for the growth rate from 2000 to 2009: 0.71-1.74 mg/L).

The test method and the test species are recommended by the test guidelines.

3.4 Test Water

Reconstituted test water prepared according to the test guidelines was used for algal cultivation and testing. Analytical grade salts were dissolved in sterile purified water to obtain the following concentrations:

Ingredients			Concentration
Macro-nutrients	$NaHCO_3$		50.0 mg/L
	KH_2PO_4		1.6 mg/L
	$MgSO_4$	× 7 H_2O	15.0 mg/L
	$MgCl_2$	× 6 H_2O	12.0 mg/L
	$CaCl_2$	× 2 H_2O	18.0 mg/L
	NH_4Cl		15.0 mg/L
Trace elements	H_3BO_3		185.0 µg/L
	$MnCl_2$	× 4 H_2O	415.0 µg/L
	$ZnCl_2$		3.0 µg/L
	$CoCl_2$	× 6 H_2O	1.5 µg/L
	$CuCl_2$	× 2 H_2O	0.01 µg/L
	Na_2MoO_4	× 2 H_2O	7.0 µg/L
	$FeCl_3$	× 6 H_2O	64.0 µg/L
	Na_2EDTA	× 2 H_2O	100.0 µg/L

The water hardness (calculated) of the test water was 0.24 mmol/L (= 24 mg/L as $CaCO_3$).

3.5 Material

50 mL Erlenmeyer flasks were used per replicate containing 15 mL of test solution. Each test flask was covered with a glass dish. The test flasks were labeled with the study number and all necessary additional information to ensure unique identification. During exposure, the test solutions were continuously stirred by magnetic stirrers.

3.6 Experimental Conditions

The test flasks were incubated in a temperature-controlled water bath at a temperature between 21 and 22 °C and illuminated by fluorescent tubes (Philips TLD 36W-1/840), installed above the test flasks. The test flasks were positioned randomly and repositioned daily. The mean measured light intensity at the level of the test solutions was approximately 7600 Lux (range: 7130 to 8240 Lux, measured at nine places in the experimental area). The light intensity over the incubation area (measured at nine places in the experimental area) was within ±15% from the average light intensity as recommended by the guideline.

3.7 Study Design

The selection of the test concentrations was based on the results of a range-finding test (non-GLP).

The following treatments were tested: Dilution 1:100, 1:32, 1:10, 1:3.2 and the undiluted filtrate with a loading rate of 100 mg/L. Additionally, a control was tested in parallel (test water without test item).

The test design included three replicates per test concentration and six replicates of the control.

The test was started using a nominal algal cell density of 10000 cells/mL. The initial cell density was selected according to the recommendations of the OECD test guideline. The algal cell density in the pre-culture was determined by an electronic particle counter (Coulter Counter®, Model ZM).

A static test design was applied. The duration of the test was 72 hours.

3.8 Dosage

Due to the low water solubility of the test item, a dispersion with the loading rate of 100 mg/L was prepared by dispersing 150.15 mg of the test item in 1500 mL of test water. The dispersion was supported by ultrasonic treatment for 15 minutes and intense stirring by a magnetic stirrer over 96 hours at room temperature in the dark, to dissolve a maximum amount of the test item in the dispersion. No auxiliary solvent or emulsifier was used.

After the stirring period, the dispersion was let to settle for 15 minutes and was then filtered through a membrane filter (Schleicher & Schuell, Type NC45, pore size 0.45 µm). The negative pressure of the filtration unit was reduced as far as possible. The test water (containing the water dissolvable part of the test item) was taken from the middle of the water column to avoid any undissolved test item in the test water. This undiluted filtrate was used as the highest concentrated test medium and as stock solution for the preparation of the test media of lower test concentrations. For the preparation of the test media of the lower test concentrations, the filtrate was diluted with test water. The test media were prepared just before the start of the test (= start of exposure).

3.9 Evaluations

3.9.1 Determination of Algal Biomass

A small volume of the algal suspension was withdrawn daily from each test flask for the measurement of the biomass, and was not replaced.

The algal biomass in the samples was determined by fluorescence measurement (BIO-TEK® Multi-Detection Microplate Reader, Model FLx800). The measurements were performed at least in duplicate.

At the end of the test, a sample was taken from the control and from the undiluted filtrate to determine a potential influence of the test item on the algal cells. The shape and size of the algal cells were visually inspected.

3.9.2 Determination of Algal Growth Inhibition and EC Values

Inhibition of algal growth was determined from the following growth parameters:

a) the specific growth rate (μ)
b) the yield (Y)

using the following equations:

a) Specific growth rate (μ):

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i}$$

where: μ_{i-j}: average specific growth rate from time i to j
X_i: biomass at time i
X_j: biomass at time j

The average growth rate over the test duration and the section-by-section growth rates (daily growth rates between the sampling times) were calculated.

Inhibition of growth rate (I_r):

$$I_r = \frac{\mu_C - \mu_T}{\mu_C} \cdot 100\%$$

where: I_r: percent inhibition in average specific growth rate
μ_C: mean value for average specific growth rate in the control group
μ_T: average specific growth rate for the treatment replicate

b) Yield (Y):

$$Y = X_j - X_0$$

where: Y: yield
X_0: biomass (nominal value) at the start of the test
X_j: biomass at time j (at the end of the test)

Inhibition of yield (I_y):

$$I_y = \frac{(Y_C - Y_T)}{Y_C} \cdot 100\%$$

where: I_y: percent inhibition of yield
Y_C: mean value for yield in the control group
Y_T: value for yield for the treatment replicate

Growth rate and yield were calculated for each test flask. The mean values for growth rate and yield were calculated for each treatment. The tabulated values represent rounded results obtained by calculation using the exact raw data.

The 72-hour EC_{50} value for the inhibition of average growth rate and yield and their 95% confidence intervals were calculated as far as possible by Probit Analysis [3, 4].

For the determination of the LOEC and NOEC, average growth rate and yield at the test concentrations were compared to the control values by Dunnett's tests [5, 6].

3.9.3 Water Quality Criteria

The pH was measured and recorded in each treatment at the start and at the end of the test. The water temperature was measured and recorded daily in an Erlenmeyer flask filled with water and incubated under the same conditions as the test flasks. The appearance of the test media was also recorded daily.

3.9.4 Analysis of the Test Item Concentrations

For measurement of the actual concentrations of the test item, duplicate samples were taken from the test media of all test concentrations at the start of the test (without algae) and at the end of the test (containing algae). At the same sampling times, duplicate samples were also taken from the control. For sampling at the end of the test, the test medium of the treatment replicates was pooled. Immediately after sampling, each sample was spiked with 90 mL MeOH.

Due to the difficult analytical detection, additional samples were prepared as follows:
At the start and the end of the test, a second set of duplicate samples (without algae) was taken of each treatment. For this, additional flasks containing the test medium without algae were incubated under the test conditions until the end of the test. These samples were not spiked with MeOH.

All samples were stored deep-frozen (at about -20 °C) immediately after sampling until analysis.

The concentrations of the test item SILRES® BS 1701 were determined in the duplicate spiked test medium samples from the dilution 1:3.2 and the undiluted filtrate from test start and test end. The samples from the less concentrated dilutions were not analyzed, since these dilutions were below the NOEC determined in this test. From the control samples, one of the duplicate samples was analyzed from the corresponding sampling times.

The analytical procedure and results are described in the corresponding appendix.

4 RESULTS AND DISCUSSION

At the start of the test, the analytically determined concentrations of the test item in the test media (dilution 1:3.2 and the undiluted filtrate) were 280 and 1200 µg/L, respectively (see analytical results and Table 2 in the corresponding appendix). During the test period of 72 hours, a decrease of test item concentration in the test media occurred. At the end of the test, the measured concentrations were both lower than the biological limit of quantification of the test item (LOQ_{bio}=10.1 µg test item/L). However, as the algae were exposed to the test item and all degradation products (produced during stirring time and the exposure period as a result of hydrolysis) the biological results were based on the loading rate of 100 mg/L and the dilutions thereof.

The influence of the test item on the growth of the algae is shown in Table 1 to Table 3 and in Figure 1 to Figure 3.

The test item had a significant inhibitory effect on the growth of the algae (average growth rate and yield) after the test period of 72 hours at the loading rate of 100 mg/L (undiluted filtrate; results of Dunnett's tests, one-sided, $\alpha = 0.05$, Table 2 and Table 3). Thus, this loading rate was determined to be the 72-hour LOELR.

The 72-hour NOELR was determined to be dilution 1:3.2 of the loading rate of 100 mg/L, since up to and including this mean measured test concentration, the growth rate and yield of the algae after 72 hours were not significantly lower than in the control.

The biological results were summarized as follows:

Parameter (0-72 h)	**Growth rate**	**Yield**
EL_{50}	>100 mg/L	
NOELR	dilution 1:3.2 (of filtrate 100mg/L)	
LOELR	100 mg/L (loading rate)	

As the water solubility was determined to be <0.25 mg/l (7) and the formation of colloidal fractions (8) in the undiluted filtrate can not be excluded, the highest loading rate tested (100 mg/L) could be above the water solubility of the test item. However, due to the characteristics of the test item, the applied test design could not be improved further.

The microscopic examination of the algal cells at the end of the test showed no difference between the algae growing in the undiluted filtrate and the algal cells in the control. The shape and size of the algal cells were obviously not affected by the test item up to at least this concentration.

In the control the biomass increased by a factor of 102 over 72 hours (Table 1). The validity criterion of increase of biomass by at least a factor of 16 within three days was fulfilled. The mean coefficient of variation of the daily growth rates in the control (section-by-section growth rates, Table 4) during 72 hours was 17%. According to the OECD test guideline, the mean coefficient of variation must not be higher than 35%. Thus, the validity criterion was fulfilled. The coefficient of variation of the average specific growth rates in the replicates of the control after 72 hours was 2.4%. According to the OECD test guideline, the coefficient of variation must not be higher than 7%. Thus, the validity criterion was fulfilled.

No remarkable observations were made concerning the appearance of the test media. All test media were clear solutions throughout the test period.

At the start and end of the test, the pH measured in the treatments was between 8.1 and 8.2 (Table 5). The water temperature during the test was between 21 and 22 °C (Table 6).

5 REFERENCES

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Taxonomic designations of the bioassay alga NIVA-CHL 1 ("*Selenastrum capricornutum*") and some related strains.
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7. TOGNUCCI,, A. (2001):
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Environmental Toxicology and Chemistry 15, 1263-1265

6 TABLES

The tabulated values represent rounded results obtained by calculation using the exact raw data.

Table 1 Biomass of Algae

Treatment / Dilution (mg/L)	Mean measured concentration (µg/L)	Rep. no.	Biomass of algae*		
			24 hours	48 hours	72 hours
Control	---	1	12.3	66.6	286.1
		2	11.6	66.8	251.0
		3	10.3	70.4	303.3
		4	11.0	70.5	254.7
		5	11.0	76.3	265.4
		6	10.4	64.5	220.0
		Mean	11.1	69.2	263.4
		SD	0.8	4.2	29.1
1:100	---	1	11.6	73.3	253.9
		2	10.3	62.1	288.0
		3	10.3	70.9	256.4
		Mean	10.7	68.7	266.1
		SD	0.7	5.9	19.0
1:32	---	1	10.8	65.2	264.4
		2	10.5	65.4	285.3
		3	10.2	73.2	272.3
		Mean	10.5	68.0	274.0
		SD	0.3	4.6	10.6
1:10	---	1	10.6	62.4	244.7
		2	10.6	68.4	299.2
		3	8.9	66.9	242.4
		Mean	10.1	65.9	262.1
		SD	1.0	3.1	32.1
1:3.2	38	1	10.8	64.1	272.5
		2	10.9	73.9	262.9
		3	11.0	71.4	253.7
		Mean	10.9	69.8	263.0
		SD	0.1	5.1	9.4
Undiluted Filtrate (100)	78	1	10.5	53.8	184.2
		2	10.3	68.5	192.0
		3	11.0	59.4	166.0
		Mean	10.6	60.6	180.7
		SD	0.4	7.4	13.3

SD: Standard deviation

*: The biomass was determined by fluorescence measurement (at least duplicate measurements per replicate) and is given as relative fluorescence units (x 10^3). At the start of the test, the initial cell density was 10000 algal cells/mL, corresponding to 2.59 x 10^3 relative fluorescence units.

Table 2 Average Growth Rates (μ)

Treatment / Dilution	Mean measured concentration	Average growth rate μ (day^{-1}) and inhibition of μ (I_r)					
		0-24 h		0-48 h		0-72 h	
(mg/L)	(μg/L)	μ	I_r (%)	μ	I_r (%)	μ	I_r (%)
Control	---	1.45	0.0	1.64	0.0	1.54	0.0
1:100	---	1.42	2.5	1.64	0.2	1.54	-0.3
1:32	---	1.40	3.7	1.63	0.5	1.55	-1.0
1:10	---	1.35	6.9	1.62	1.5	1.54	0.1
1:3.2	38	1.44	1.2	1.65	-0.3	1.54	-0.1
Undiluted Filtrate	78	1.41	3.0	1.57	4.2	1.41*	8.1

*: mean value significantly lower than in the control
(according to Dunnett's-tests, one-sided, $\alpha = 0.05$)

Table 3 Yield (Y)

Treatment / Dilution	Mean measured concentration	Yield Y (x 10^3) and inhibition of Y (I_y)					
		0-24 h		0-48 h		0-72 h	
(mg/L)	(μg/L)	Y	I_y (%)	Y	I_y (%)	Y	I_y (%)
Control	---	8.5	0.0	66.6	0.0	260.8	0.0
1:100	---	8.1	4.6	66.2	0.7	263.5	-1.0
1:32	---	7.9	7.1	65.4	1.8	271.4	-4.1
1:10	---	7.5	12.3	63.3	4.9	259.5	0.5
1:3.2	38	8.3	2.5	67.2	-0.9	260.5	0.1
Undiluted Filtrate	78	8.0	5.8	58.0	12.9	178.1*	31.7

*: mean value significantly lower than in the control
(according to Dunnett's-tests, one-sided, $\alpha = 0.05$)

Table 4 Section-by-Section Growth Rates

Treatment / Dilution	Mean measured concentration	Section-by-section growth rates (day^{-1}) and inhibition of the growth rates (I_r)					
		0-24 h		24-48 h		48-72 h	
(mg/L)	(µg/L)	µ	I_r (%)	µ	I_r (%)	µ	I_r (%)
Control	---	1.45	0.0	1.83	0.0	1.33	0.0
1:100	---	1.42	2.5	1.86	-1.6	1.35	-1.6
1:32	---	1.40	3.7	1.87	-2.0	1.40	-4.7
1:10	---	1.35	6.9	1.88	-2.9	1.38	-3.2
1:3.2	38	1.44	1.2	1.86	-1.4	1.33	0.4
Undiluted Filtrate	78	1.41	3.0	1.74	5.0	1.10	17.8

Table 5 pH Values in the Treatments

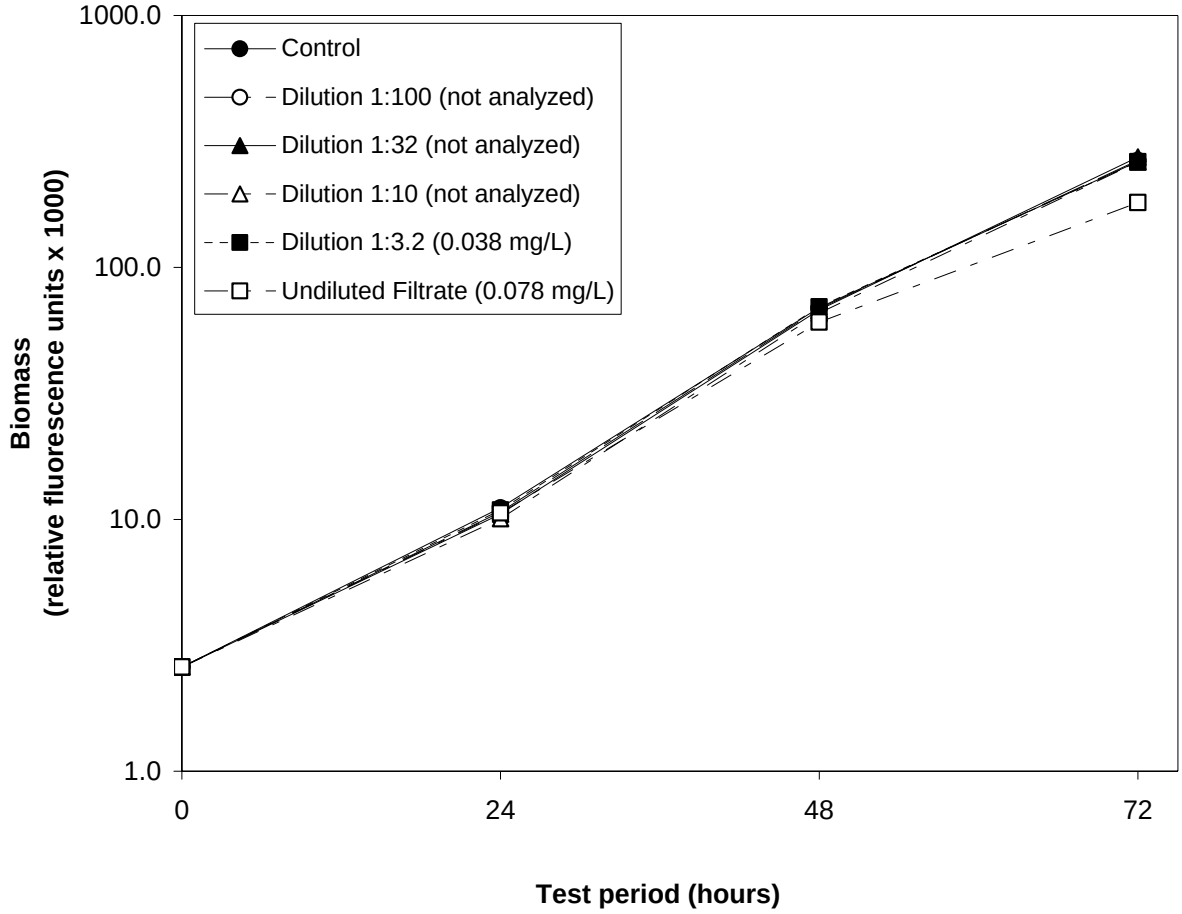
Treatment / Dilution	pH values	
(mg/L)	Start	End
Control	8.1	8.1
1:100	8.2	8.2
1:32	8.2	8.2
1:10	8.1	8.2
1:3.2	8.1	8.2
Undiluted Filtrate	8.1	8.1

Table 6 Water Temperature during the Test Period

	Temperature (°C)
Day 0 (Start)	21
Day 1	21
Day 2	22
Day 3 (End)	22

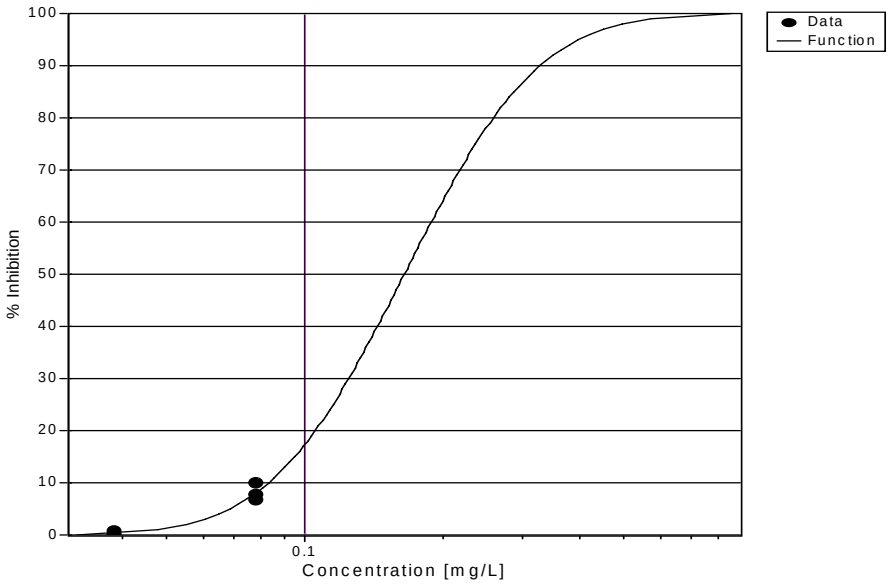
7 FIGURES

Figure 1 Growth Curves of the Algae over the Test Duration of 72 Hours



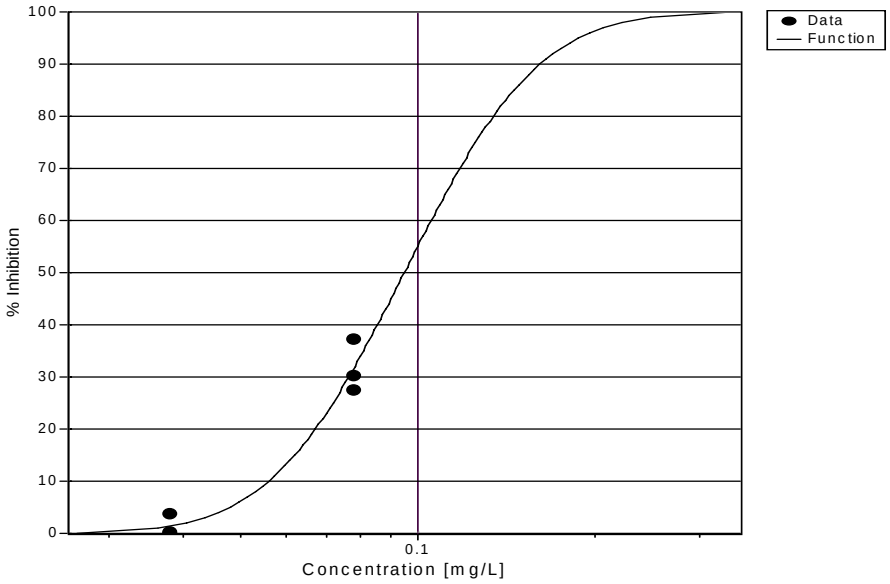
(Treatment/Dilution and in () mean measured concentrations of the test item)

Figure 2 Concentration-Effect Relationship of Average Growth Rates after 72 Hours



(mean measured concentrations of the test item)

Figure 3 Concentration-Effect Relationship of Yield after 72 Hours



(mean measured concentrations of the test item)

APPENDIX I – ANALYTICAL INVESTIGATIONS

Analytical Investigations

CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 MATERIAL AND METHODS

The analytical method was developed at the Harlan Laboratories[1].

1.1 Test Item

The test item described in the main body of this report was also used as analytical standard.

1.2 Analytical Procedure

1.2.1 Storage

Aliquots of 10 mL of the samples were diluted to 100 mL using methanol, stored deep-frozen and protected from light until analysis was performed.

1.2.2 Reagents and Solvents

Water	in-house prepared by a water purification system (Millipore)
Test water	as described in the main body of this report
Methanol > 99.8%	J.T. Baker, No. 8402

1.2.3 Preparation of Calibration Solutions

The test item (13.29 mg) was dissolved in methanol and made up to the mark in a 50 mL volumetric flask to prepare a stock solution with a concentration of 266 mg/L. Thereof, 0.19 mL were diluted to 10 mL using methanol/water (v/v; 9/1) to obtain a working solution with a concentration of 5050 µg/L. Defined volumes of this stock solution and of higher concentrated calibration solutions were diluted with methanol/water (v/v; 9/1) to obtain calibration solutions of the test item in the range of 1.01 to 253 µg/L. These solutions were used to calibrate the analytical system.

[1] These experiments were not performed according to the regulations of GLP. The raw data are archived under Harlan Laboratories study C17058.

1.2.4 Preparation of Spiked Test Water Samples

To demonstrate the validity of the method, untreated test water was spiked with the test item. Therefore, the test item (13.2 mg) was dissolved in methanol and made up to the mark in a 50 mL volumetric flask to prepare a stock solution with a concentration of 264 mg/L. Thereof, 4 mL were made up to 10 mL with methanol/water (v/v; 9/1) to obtain fortification solution 1 with a concentration of 106 mg/L of the test item. Thereof, 0.4 mL were made up to 10 mL with methanol/water (v/v; 9/1) to obtain fortification solution 2 with a concentration of 10.6 mg/L of the test item. Defined volumes of these fortification solutions were diluted with test water to obtain spiked samples with concentrations of 211 µg/L (from fortification solution 1) and 1056 µg/L (from fortification solution 1). These solutions were freshly prepared in duplicate. Aliquots of 1 mL were diluted to 10 mL using methanol giving a sample preparation factor of ten. In addition, test water without the test item was analyzed (analytical blank). Aliquots of the final solutions were analyzed centrifuged (3600 rpm, 5 min) and non-centrifuged by GC/MS.

1.2.5 Preparation of Samples

Treatment samples and control samples were thawed at 30 °C for 1.5 hours and shaken manually to obtain homogeneous sample solutions. Aliquots of the diluted sample solutions (sample preparation see caption 1.2.1) were analyzed by GC/MS. The samples from day 3 were centrifuged (3600 rpm, 5 min) before analysis due to algal growth.

1.2.6 GC-Conditions

Gas Chromatograph:	AGILENT 6890
Detector:	MS 5973
Column:	VF WAX MS (Varian) (30 m; 0.25 mm inner diameter; 0.25 µm film thickness)
Carrier Gas:	Helium Column flow: 1.2 mL/Minute Constant flow
Temperature Program:	Injector: 300 °C Oven: 50 °C, for 1 Minute with 25 °C/Minute to 250 °C
Injection Mode:	Pulsed Splitless Injection Volume: 1 µL Insert: Glasswool
Detector Parameters:	Ion Source: 230 °C Scan Mode: Sim
Ionization Mode:	EI
Scan Mode:	SIM m/z: 119 ± (Qualifier) **m/z: 163 ± (Quantifier)** m/z: 205 ± (Qualifier)
Retention Time:	Approximately 4.15 minutes

1.2.7 Data Evaluation and Quantification

Injected samples were quantified by peak areas with reference to the respective calibration curve. The latter was obtained by correlation of the average peak area of the calibration solutions injected in duplicates and to their corresponding concentration in µg/L. The correlation was performed using a linear function (for an example see Figure 1 and Table 1). From the calibration curve, the concentration x of the test item in an injected sample was calculated by equation (1):

$$x = \frac{y - a}{b}, \text{ by weighting } 1/y \qquad (1)$$

where: x: concentration of the test item in injected sample [µg/L]
y: peak area of the test item in injected sample [counts]
a: y-axis intercept
b: slope

The concentration of the test item in a sample was calculated by equation (2):

$$c = x \cdot F \qquad (2)$$

where: c: concentration of the test item in the sample [µg/L]
x: amount of the test item in injected sample determined by equation (1) [µg/L]
F: sample preparation factor (F = 10)

The recovery rate was calculated by equation (3):

$$R = \frac{c}{c_{nom}} \cdot 100\% \qquad (3)$$

where: R: recovery rate
c: concentration of the test item in the sample determined by equation (2) [µg/L]
c_{nom}: nominal concentration of the test item in the sample [µg/L]

2 RESULTS AND DISCUSSION

The results obtained for the concentrations of SILRES® BS 1701 in the test medium are presented in Table 2, and those for the spiked test water samples in Table 3.

An example of the calibration data for the calibration solutions of the test item is given in Table 1 and Figure 1. The R^2 fits of the calibration curves used were 0.9996 and 0.9958. This reflects the linearity of the analytical system within the calibration range of 1.01 - 253 µg test item/L.

Concurrent with the sample analysis, recoveries of spiked test water samples at relevant concentrations (211 and 1056 µg test item/L) were performed in duplicate. The average recoveries for the non-centrifuged samples were found to be 95% and 115% of the spiked values, with an overall mean of 105% (n = 4). The average recoveries for the centrifuged samples were found to be 95% and 124% of the spiked values, with an overall mean of 110% (n = 4).No correction for the recovery rate was made.

The limit of quantification for the test item in the injected solution (LOQ_{ana}) was derived from the lowest calibration solution, which fits into the calibration curve: the value is 1.01 µg test item/L. The limit of quantification for the test item in treatment samples (LOQ_{bio}) was 10.1 µg test item/L taking into account a sample preparation factor of ten.

The biological control samples and an analyzed analytical blank (test water) did not significantly affect the chromatogram at the retention time of the test item.

The average concentrations found in the treatment samples were 283 µg/L and 1189 µg/L (day 0) and below the LOQ_{bio} (day 3).

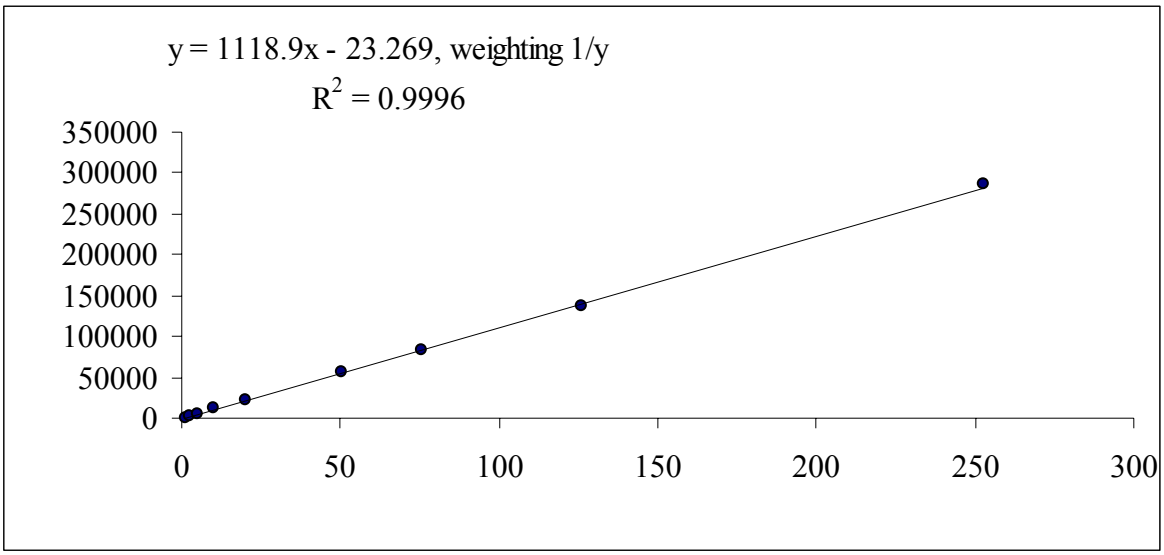
Typical chromatograms are shown in Figure 2 to Figure 7.

3 TABLES AND FIGURES

Table 1 Example of Calibration Data of Test Item

Concentration of Test Item [µg/L]	Signal [counts]	Mean Signal [counts]	Deviation of the Calculated Value from the Effective Value [%]
1.01	1146	1092	-1.4
	1037		
2.53	3077	2930	4.5
	2782		
5.05	5107	5551	-1.3
	5995		
10.1	11944	11089	-1.7
	10234		
20.2	25966	23044	2.1
	20121		
50.5	62828	56688	0.4
	50547		
75.8	95342	84086	-0.8
	72830		
126	155702	137040	-3.0
	118378		
253	318946	287062	1.6
	255178		

Figure 1 Graphical Interpretation of the Calibration Data



X-Axis: Test Item Concentration in µg/L; Y-Axis: Signal in Area Counts

Table 2 Results Obtained for the Concentrations of the Treatment Samples

Age [day]	Sample ID	Loading Rate of 100 mg Test Item/L	Measured Concentration of Test Item x			Sample Preparation Factor F	Determined Average Concentration of Test Item c [µg/L]
			Sample 1 [µg/L]	Sample 2 [µg/L]	Average [µg/L]		
0	A1	Control	$<LOQ_{ana}$	-	n.a.	10	n.a.
	A9/10	Dilution 1:3.2	28.3	28.3	28.3	10	283
	A11/12	Undiluted Filtrate	119	119	119	10	1189
3	A25	Control	$<LOQ_{ana}$	-	n.a.	10	n.a.
	A33/34	Dilution 1:3.2	$<LOQ_{ana}$	$<LOQ_{ana}$	n.a.	10	$<LOQ_{bio}$
	A35/36	Undiluted Filtrate	$<LOQ_{ana}$	$<LOQ_{ana}$	n.a.	10	$<LOQ_{bio}$

LOQ_{ana} = 1.01 µg test item/L
LOQ_{bio} = 10.1 µg test item/L
n.a. = not applicable

The tabulated values of the samples represent rounded results obtained by calculation using the exact raw data.

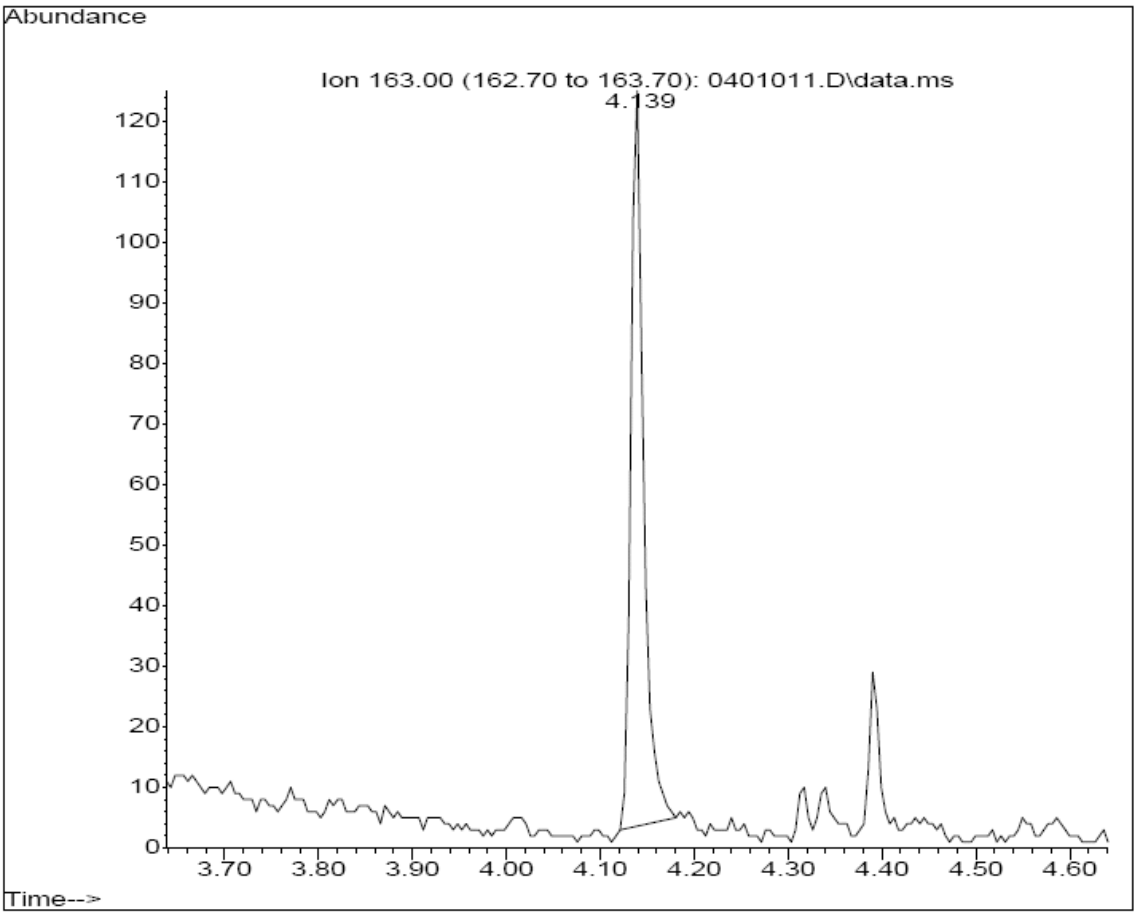
Table 3 Results Obtained for the Concentrations of the Spiked Test Water Samples

Sample ID	Nominal Concentration of Test Item c_{nom} [µg/L]	Measured Concentration in the Spiked Sample x [µg/L]	Sample Preparation Factor F	Concentration Test Item Determined in the Spiked Sample c [µg/L]	Recovery Rate R [%]	Average [%]
Non-Centrifuged Samples						
AZ000	0	<LOQ_{ana}	10	n.a.	n.a.	-
AZ9	211	19.5	10	195	92	
AZ10	211	20.5	10	205	97	95
AZ11	1056	123	10	1230	117	
AZ12	1056	121	10	1207	114	115
					Overall mean:	105
Centrifuged Samples						
AZ000	0	<LOQ_{ana}	10	n.a.	n.a.	-
AZ9	211	20.1	10	201	95	
AZ10	211	20.2	10	202	95	95
AZ11	1056	130	10	1301	123	
AZ12	1056	132	10	1321	125	124
					Overall mean:	110

LOQ_{ana} = 1.01 µg test item/L
n.a. = not applicable

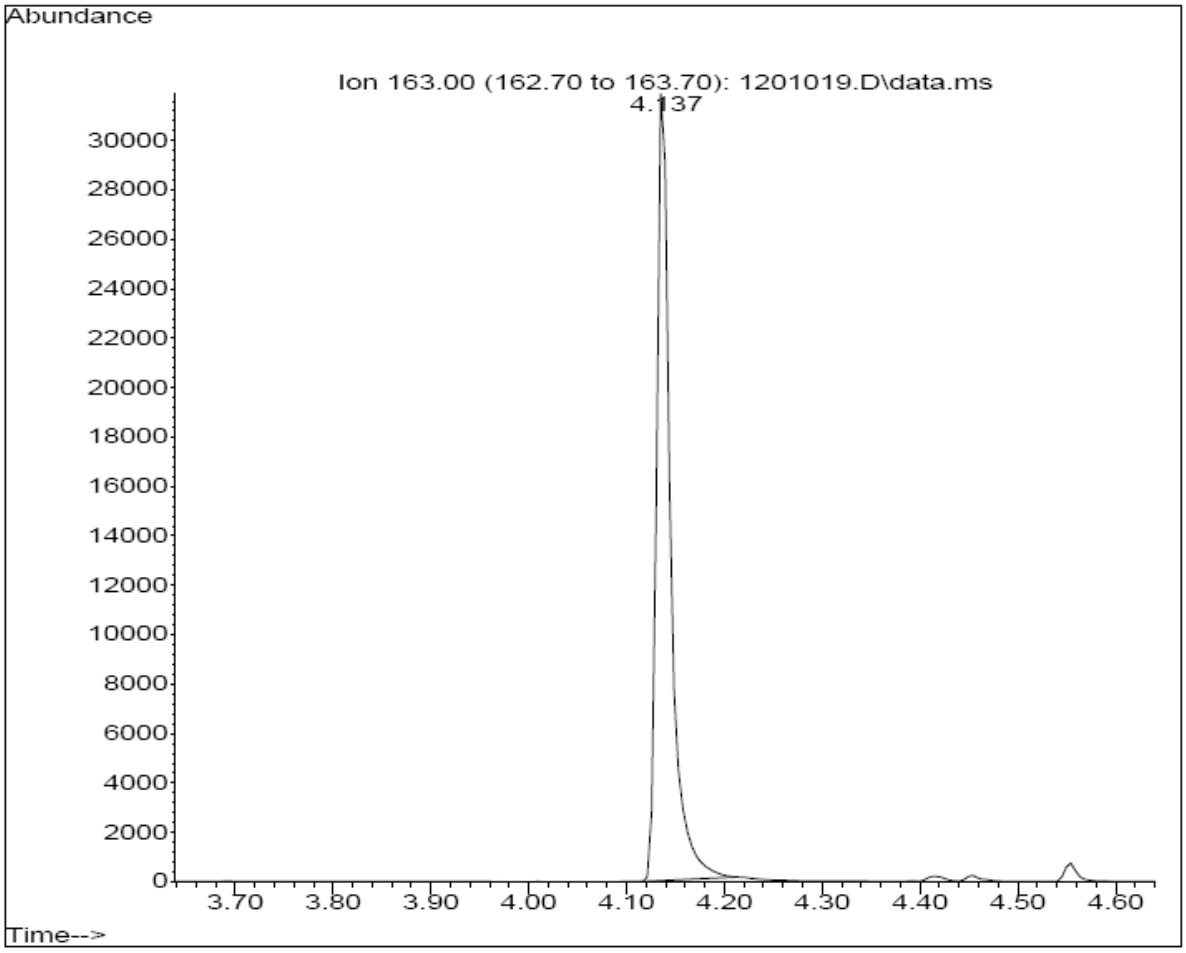
The tabulated values of the samples represent rounded results obtained by calculation using the exact raw data.

Figure 2 Chromatogram of Calibration Solution (Low-Level)



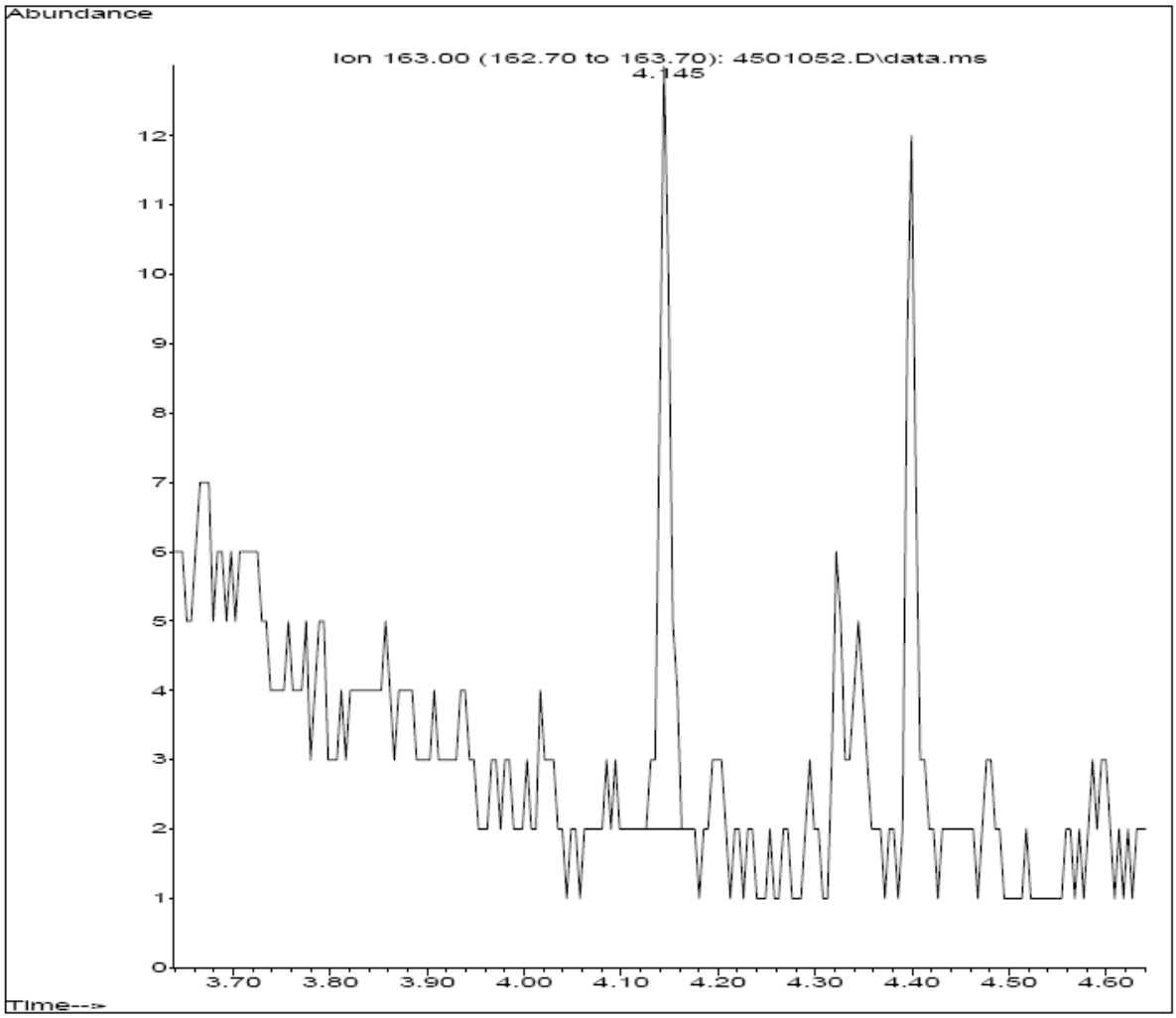
Concentration: 1.01 µg test item/L

Figure 3 Chromatogram of Calibration Solution (High-Level)



Concentration: 253 µg test item/L

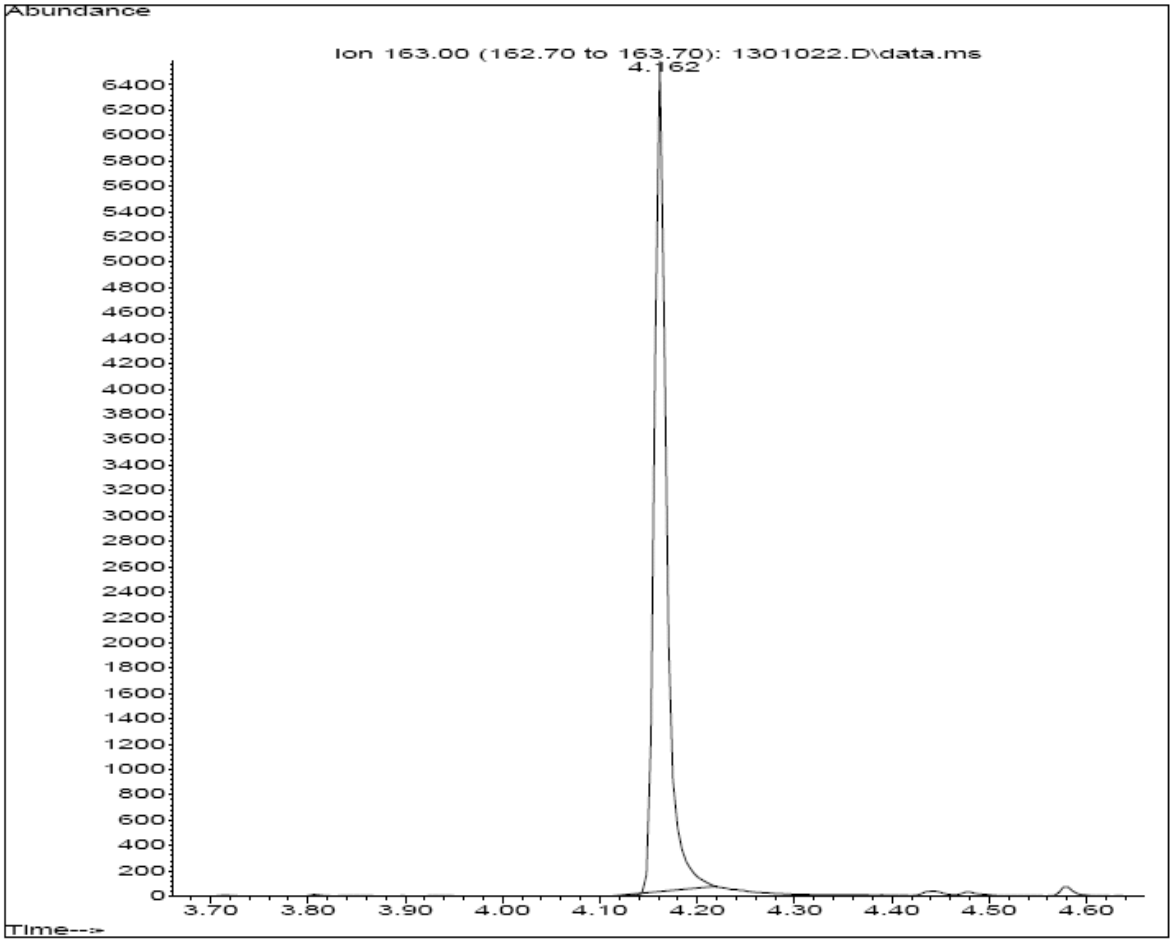
Figure 4 Chromatogram of Biological Control Sample



Sample ID: A1
Sampling day: 0

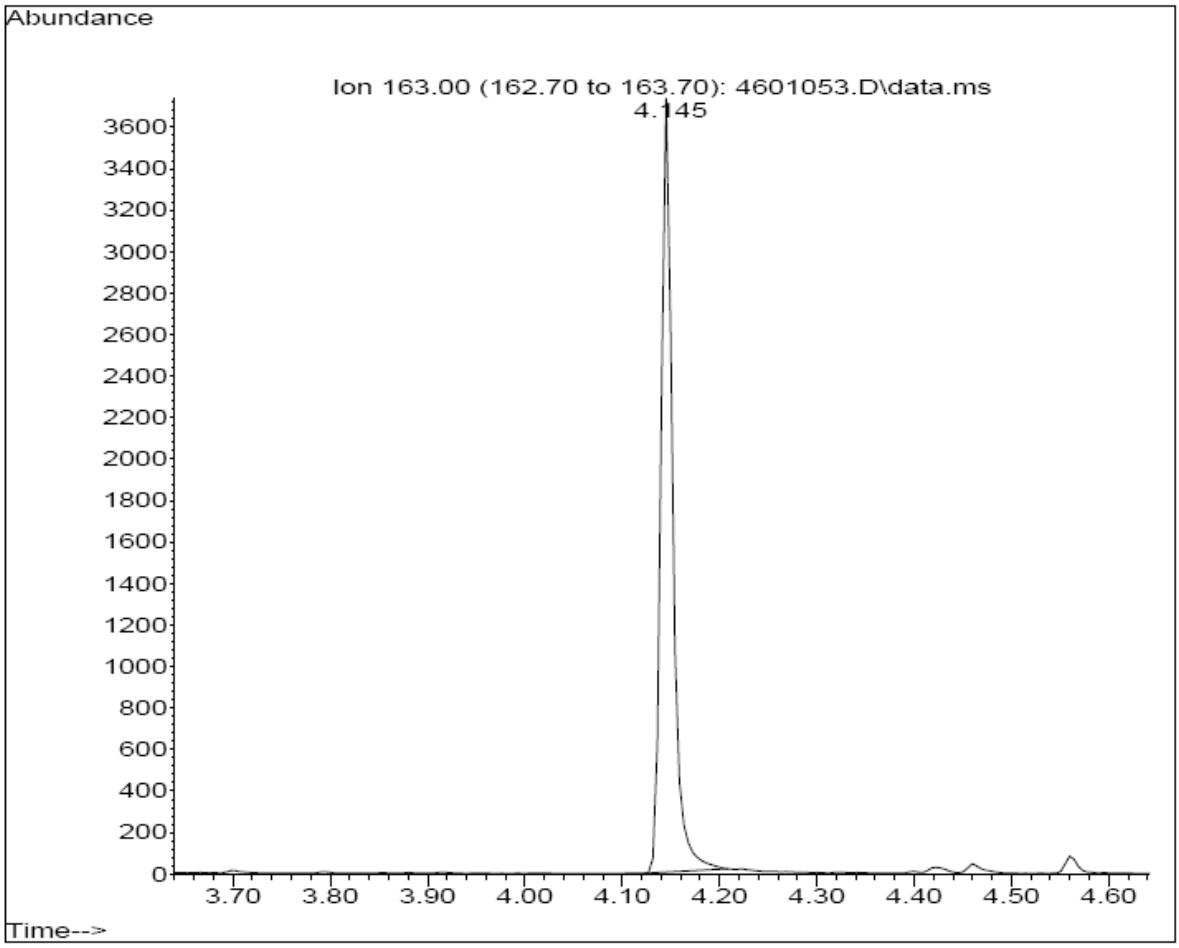
(The measured concentration in the injected solution was below the LOQ_{ana} of 1.01 µg test item/L)

Figure 5 Chromatogram of Spiked Test Water Sample



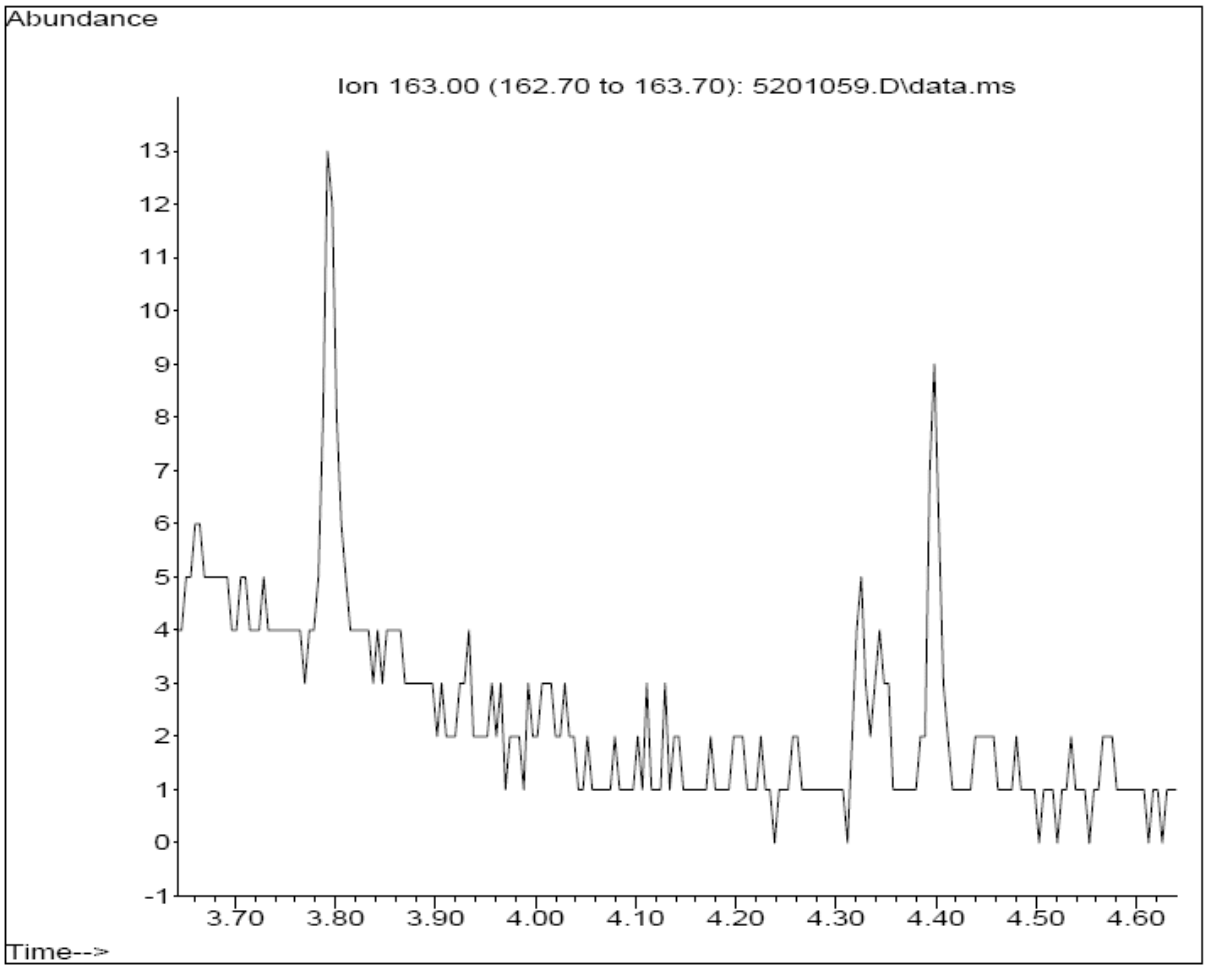
Sample ID: AZ11 Non-Centrifuged
(The measured concentration in the injected solution was 123 µg test item/L)

Figure 6 Chromatogram of Non-Aged Treatment Sample



Sample ID: A9
Sampling day: 0
(The measured concentration in the injected solution was 28.3 µg test item/L)

Figure 7 Chromatogram of Aged Treatment Sample



Sample ID: A33
Sampling day: 3
(The measured concentration in the injected solution was below the LOQ_{ana} of 1.01 µg test item/L)

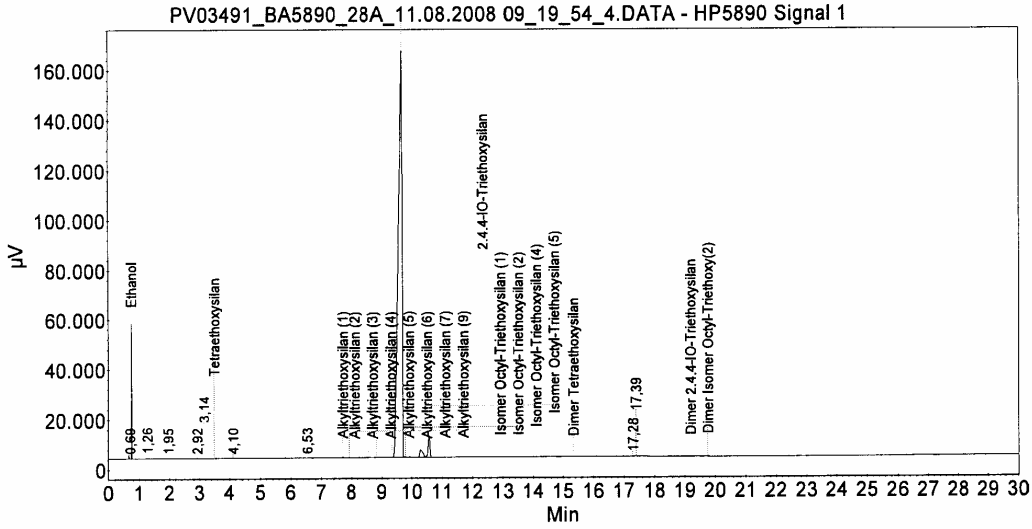
APPENDIX II – CERTIFICATE OF ANALYSIS

WACKER

Wacker Chemie AG, WL-C-A-B
Betriebsanalytik / Quality Control

Probenbezeichnung:	109807 - SILRES BS 1701, KH07241
Datenfile:	PV03491_BA5890_28A_11.08.2008 09_19_54_4
Auftragsdaten:	Auftrag Q-00050236-05AUG2008, Probe 200603, Test 552899, Prüflos 000003258399

Analyse am:	11.08.2008 09:21:55	Bearbeiter:	Spitaler Maria
Methode:	PV03491_BA5890_28A	Runtime:	30,00 min
Gerät:	BA5890_28	Injektor:	HP5890 Front Injector
Säulenparameter:	DB-1 Nr.54, 30m, 0,53mm, 1,5µm	Detektor:	WLD
Einspritzmenge:	2 stop(s)	Probentyp:	Blank/Unknown
Kalibrationstyp:	Normalization	Multiplikator:	1,000
Probengewicht:	N.A.		
ISTD-Gewicht:	N.A.		



Index	Time [Min]	RF	Area [µV.Min]	Area % [%]	Quantity [%]	Name
24	0,69	1,000	1,7801	0,007	0,007	
1	0,75	0,646	417,4172	1,707	1,110	Ethanol
2	1,26	1,000	6,1168	0,025	0,025	
3	1,95	1,000	0,0132	0,000	0,000	
4	2,92	1,000	0,2074	0,001	0,001	
5	3,14	1,000	7,5002	0,031	0,031	
6	3,49	1,000	0,8063	0,003	0,003	Tetraethoxysilan
7	4,10	1,000	1,4979	0,006	0,006	
8	6,53	1,000	4,2330	0,017	0,017	
25	7,72	1,000	14,0234	0,057	0,058	Alkyltriethoxysilan (1)
26	7,92	1,000	2,3680	0,010	0,010	Alkyltriethoxysilan (2)
9	8,09	1,000	2,5757	0,011	0,011	Alkyltriethoxysilan (3)
10	8,18	1,000	3,3558	0,014	0,014	Alkyltriethoxysilan (4)
11	8,59	1,000	16,0715	0,066	0,066	Alkyltriethoxysilan (5)
12	8,70	1,000	1,6582	0,007	0,007	Alkyltriethoxysilan (6)
13	8,82	1,000	18,6465	0,076	0,077	Alkyltriethoxysilan (7)
14	9,34	1,000	22,3271	0,091	0,092	Alkyltriethoxysilan (9)
15	9,68	1,000	22866,1720	93,536	94,105	2.4.4-IO-Triethoxysilan
16	9,78	1,000	16,5927	0,068	0,068	Isomer Octyl-Triethoxysilan (1)
17	9,92	1,000	37,3919	0,153	0,154	Isomer Octyl-Triethoxysilan (2)
18	10,29	1,000	386,7518	1,582	1,592	Isomer Octyl-Triethoxysilan (4)

22.09.2008 09:15:18

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WACKER

Wacker Chemie AG, WL-C-A-B
Betriebsanalytik / Quality Control

Probenbezeichnung:	109807 - SILRES BS 1701, KH07241
Datenfile:	PV03491_BA5890_28A_11.08.2008 09_19_54_4
Auftragsdaten:	Auftrag Q-00050236-05AUG2008, Probe 200603, Test 552899, Prüflos 000003258399

Analyse am:	11.08.2008 09:21:55	Bearbeiter:	Spitaler Maria
Methode:	PV03491_BA5890_28A	Runtime:	30,00 min
Gerät:	BA5890_28	Injektor:	HP5890 Front Injector
Säulenparameter:	DB-1 Nr.54, 30m, 0,53mm, 1,5µm	Detektor:	WLD
Einspritzmenge:	2 stop(s)	Probentyp:	Blank/Unknown
Kalibrationstyp:	Normalization	Multiplikator:	1,000
Probengewicht:	N.A.		
ISTD-Gewicht:	N.A.		

Index	Time [Min]	RF	Area [µV.Min]	Area % [%]	Quantity [%]	Name
19	10,57	1,000	584,9731	2,393	2,407	Isomer Octyl-Triethoxysilan (5)
20	15,33	1,000	4,7741	0,020	0,020	Dimer Tetraethoxysilan
21	17,28	1,000	5,2486	0,021	0,022	
22	17,39	1,000	14,6299	0,060	0,060	
23	19,20	1,000	8,0485	0,033	0,033	Dimer 2.4.4-IO-Triethoxysilan
27	19,77	1,000	1,1196	0,005	0,005	Dimer Isomer Octyl-Triethoxy(2)
Total			24446,3007	100,000	100,000	

	Name	Quantity [%]
	Alkyltriethoxysilan	0,33
	Isomer Octyl-Triethoxysilan	4,22
	Dimer Isomer Octyl-Triethoxy	0,00
	Wirkstoffgehalt	98,70
Total		

22.09.2008 09:15:18

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